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Quantitative Fluorometric Analysis of Plant and Microbial Chitosanases[1]

Wolfgang F. Osswald, Roy E. McDonald, Randall P. Niedz, Jeffrey P. Shapiro, and Richard T. Mayer[2]
U.S. Horticultural Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture
2120 Camden Road, Orlando, Florida 32803-1419

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A quantitative fluorometric assay for chitosanase activity in bacterial and plant tissues was developed. The assay can be conducted with either finely milled preparations of chitosan in suspension or dissolved chitosan; activity is based on measurements of glucosamine (GlcN) or oligomers of GlcN. GlcN is detected fluorometrically after reaction with fluorescamine with detection in the nanomole range. Fluorescence measurements of chitosanase activity and radioassay of chitinase in commercial preparations of chitinase from *Streptomyces griseus* revealed that both activities were present. Specific activities for the *S. griseus* chitosanase using suspended and soluble chitosans were respectively 1.24 and 6.4 μmol GlcN · min^{-1} · mg protein^{-1}. Specific activity of the *S. griseus* chitinase was 0.98 μmol GlcN · min^{-1} · mg protein^{-1}. Sweet orange callus tissue was tested for chitosanase and chitinase activity. It was necessary to remove small amine-containing molecules from the callus preparations before chitosanase activity could be assayed. The specific activity for chitinase and chitosanase in desalted extracts of nonembryogenic Valencia sweet orange callus tissue was determined to be 18.6 and 89.4 nmol GlcN · min^{-1} · mg protein^{-1}, respectively.

There has been a great deal of interest generated in pathogenesis-related proteins since their discovery in 1970. Stress and pathogen infection often result in the induction of pathogenesis-related proteins in plants (1) and their presence is often associated with plant defense systems. Five groups of tobacco pathogenesis-related proteins have been identified and are used for nomenclature purposes (2). Pathogenesis-related proteins occur widely in a number of different plants (1,2).

Chitinases and chitosanases are considered part of the pathogenesis-related protein family (1–5). Chitinases and chitosanases degrade chitin (a polymer of *N*-acetylglucosamine; GlcNAc) and chitosan (deacetylated chitin), respectively, into saccharides and oligosaccharides. Although plants themselves contain neither chitin nor chitosan, many plant pests do. For example, fungi contain both chitin and chitosan (6). These enzymes are not unique to plants; it has been known for sometime that chitinases are important to arthropods and are essential during the molting process (7,8) and that microbes can produce both chitinases and chitosanases (3). The possibility exists that if chitinase levels in plants could be raised to sufficiently high levels, resistance to plant pathogens such as fungi would ensue. Bedbrook *et al.* (9) have successfully transferred bacterial chitinase genes into plants and have observed expression of the genes. It is conceivable that chitosanases could be utilized in a similar manner.

Studies of chitinases and chitosanases have been complicated by the fact that some chitinases may possess chitosanase activities (10) while others do not (3,4). Much is known about chitinases, perhaps because of the availability of facile radic assays (11). Knowledge about chitosanases has been hampered by the fact that assays have been dependent on colorimetric methods, which are not highly sensitive (12). Recently, procedures for the qualitative tests for chitinases and chitosanases after polyacrylamide gel electrophoresis have become available (4,13). These methods have assisted in understanding the roles of chitinases and chitosanases in plants; however, what is really needed to gain full knowledge is a sensitive, quantitative assay for chitosanase.

This laboratory previously developed a quantitative method for the detection of amine-containing carbohy-

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[2] To whom correspondence should be addressed.

0003-2697/92 $5.00

drates (14). The method is based on the reaction of fluorescamine with the amino sugar to produce a highly fluorescent product. We have now adapted this method for the quantitative assay of chitosanases.

MATERIALS AND METHODS

Chemicals

Crab (Lot 89F0383) and krill (Lot 40F02741) chitosans, thiophenol, dimethyl sulfoxide, and acetic anhydride were purchased from Sigma Chemical Co. (St. Louis, MO). A "crustacean" chitosan preparation (Lot 22742) and fluorescamine (Fluram) were obtained from Fluka (Buchs, Switzerland). Shrimp chitosan was purchased from Atomergics Chemetals Corp. (Farmingdale, NY; Lot L0729). A completely deacetylated crab chitosan was obtained as a gift from Katakura Chikkarin Co., Ltd. (Ibaraki, Japan). ^{3}H-labeled acetic anhydride (sp act 50 mCi/mmol) was obtained from NEN (Wilmington, DE).

Chitinase Assay

Chitinase activity was measured according to Molano *et al.* (11) using tritiated chitin prepared by acetylation of shrimp shell chitosan with ^{3}H-labeled acetic anhydride. The specific activity of the prepared chitin was determined after acid hydrolysis (15) via fluorometric analysis (14); the specific activity was 605 μCi · mmol^{-1} GlcNAc. Radioassays consisted of 100 μl [^{3}H]chitin (0.1 μmol GlcNAc) suspension, 10, 30, 60, or 100 μl enzyme extract, and 100 mM phosphate buffer (pH 6.5) to make a total volume of 300 μl. Reactions were initiated with enzyme and the reaction was allowed to proceed for 30 min at 37°C in a gyratory water-bath shaker. The reactions were terminated with 300 μl of 1 M trichloroacetic acid (TCA)[3] and subsequently centrifuged at 14,000*g* for 10 min. Aliquots (100 μl of the supernatants) were mixed with 5 ml scintillation cocktail (Ecoscint A, National Diagnostics, Manville, NJ) in scintillation vials and the radioactivity was measured in a liquid scintillation spectrometer (1219 Rackbeta, LKB Instruments, Gaithersburg, MD). One unit of chitinase activity is defined as the release of 1 nmol GlcNAc · min^{-1}.

Chitosanase Assay Using Chitosan Suspensions

Chitosanase reaction mixtures consisted of 100 μl chitosan suspension (4 mg · ml^{-1} of 100 mM sodium phosphate buffer, pH 6.5); 10, 30, 60, or 100 μl enzyme; and 100 mM sodium phosphate buffer (pH 6.5) to make a final volume of 300 μl. All chitosan preparations used for activity measurements were milled to 60 mesh in a Wiley mill (Model 3383-L60, Thomas Scientific, Philadelphia, PA). Chitosan concentrations used in the reactions were saturating. Reactions were initiated by addition of enzyme and allowed to proceed for 30 min at 37°C in a gyratory water-bath shaker. Reactions were terminated by addition of 300 μl of 1 M TCA. Two milliliters of a 1 M Na_2HPO_4 solution and 0.4 ml of a 0.6 N NaOH solution were mixed with the reaction volume. After centrifugation (14,000*g*, 10 min), 1 ml of the supernatant was pipetted into 1 ml of 0.2 M phosphate buffer (pH 8.8). Amino sugars were derivatized by addition of 50 μl of fluorescamine (3 mg · ml^{-1}) in either acetonitrile or acetone. Fluorescence was measured using an SLM-Aminco SPF 500C spectrofluorometer (Urbana, IL) at excitation and emission wavelengths of 395 and 493 nm, respectively. Chitosanase activity was calculated on the basis of GlcN equivalents using a GlcN standard curve generated under the same conditions as those used for the assays. Controls consisted of the complete reaction mixtures stopped immediately after addition of enzyme. Because proteolytic action may generate peptides and amino acids that could interfere with the measurement of amino sugars, control values that would account for protease contributions were determined from reactions mixtures that did not contain chitosan. A third control containing only buffer and chitosan was prepared to determine any fluorescence contributed by chitosan. One unit of chitosanase activity is defined as the release of 1 nmol GlcN · min^{-1}.

Chitosanase Assay Using Soluble Chitosan

A stock chitosan solution was prepared by dissolving 400 mg of the desired chitosan in 100 ml of 50 mM acetic acid. The pH of the stock solution was adjusted to 5 using KOH. Reactions were conducted at 37°C in a gyratory shaker. Generally the reaction mixtures contained 100 μl of 100 mM acetate buffer (pH 5) and 100 μl chitosan stock. In some instances 100 mM sodium phosphate buffer, pH 6.5, was used. Chitosanase (100 μl; 0.34 or 3 μg *S. griseus* protein) was used to initiate the reactions. Reactions were terminated by the addition of 1 M KOH (50 μl). The reaction tubes were transferred to ice for 30 min to aid the precipitation of chitosan and then centrifuged for 10 min at 10,000*g*. Aliquots (100 μl) of the supernatants were transferred to test tubes containing 1.9 ml of 0.7 M sodium phosphate buffer (pH 8.4). Fluorescamine was then added and the fluorescence measured as described above.

Enzyme Preparations

Commercial preparations of lyophilized chitinase (60% protein) from *S. griseus* were from Sigma (Lot C-1525). Chitinase and chitosanase were also obtained from plant tissues. Nonembryogenic Valencia sweet orange callus tissue (2 g fresh weight) was homogenized in

[3] Abbreviations used: TCA, trichloroacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

4 ml ice-cold 100 mM acetate buffer (pH 5) with a Brinkmann Polytron homogenizer (PT 10/35; Westbury, NY) equipped with a PTA 10S generator for 2 min. The homogenate was transferred to an ice-cold Ten Broeck Teflon–glass homogenizer and further homogenized for 20 s. The homogenate was then centrifuged at 10,000*g* at 5°C for 15 min. An aliquot (2.5 ml) of the supernatant was applied to a NAP-G25 (Pharmacia, Piscataway, NJ) column (5 × 1.5 cm) and eluted using 3.5 ml of 100 mM phosphate buffer (pH 6.5); this was done to eliminate low-molecular-weight compounds (e.g., amino acids and peptides) that may interfere with the assay.

pH Effects

Acetate (pH 3–6) and phosphate (pH 6–8) buffers at 100 mM concentrations were used. Solutions of shrimp shell chitosan (18% acetylated) were employed as the substrate as described above except that 3 μg of *S. griseus* protein was used. Reactions were run for 20 min. The activities were normalized because of differences in activity due to the buffers.

Protein Determination

Protein determinations followed the method of Bradford (16). A standard curve in the range of 0–20 μg was constructed using bovine serum albumin as the standard.

Preparation of 100% Deacetylated Chitosan

Two fully deacetylated chitosan samples were used in experiments. The first was a gift from Katakura Chikkarin Co., Ltd. (Ibaraki, Japan) and was prepared from crab shell. The second was prepared from shrimp shell chitosan (Atomergics Chemetals Corp.) essentially as described by Domard and Rinaudo (17).

ir Spectra of Chitosan Samples

The degree of acetylation of the chitosan samples was determined by ir spectroscopy using the method of Domard and Rinaudo (17). Infrared spectra were run on a Nicolet 740 FTIR (Houston, TX) using an advanced diffuse reflectance device. Samples were heated at 105°C for 1 h over drierite ($CaSO_4$) and then immediately placed in the nitrogen-purged spectrometer sample cavity. The spectra are the result of 128 scans at 4 cm^{-1} resolution. All spectra were converted to the absorbance mode, smoothed by a 15-point smoothing process, and baseline corrected to zero absorbance.

Citrus Tissue Cultures

A nonembryogenic cell line (Val88-1) was developed from immature fruit vesicles of *Citrus sinensis* (L.) Osbeck cultivar "Valencia." Immature fruit were collected 6 weeks postpollination in the spring of 1988 in Orlando, Florida, and the vesicles were removed and cultured on Murashige and Tucker's (18) basal medium supplemented with 1 μM 6-benzylaminopurine, 2.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and 100 mg · $liter^{-1}$ casein hydrolysate to induce callus. The vesicle callus cultures were subcultured every 21 days and grown in a growth cabinet under low light (15–20 μE · m^{-2} · s^{-1}), provided by cool-white fluorescent lamps, constant 27°C, and a 4-h photoperiod. After 8 months of selection, a rapidly growing callus that was only slightly friable was obtained. For maintenance, Val88-1 was transferred to the same culture medium but with the 2,4-D reduced to 1 μM and the subculture period increased to 28 days.

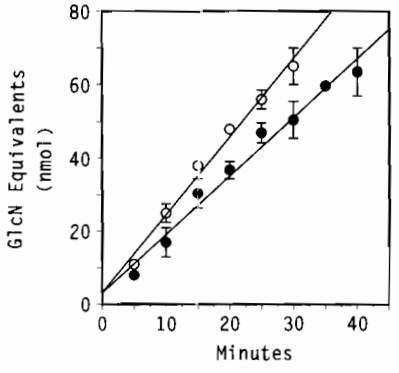

FIG. 1. Time curve of *Streptomyces griseus* chitinase activity using chitosan as the substrate. The assay was conducted as described under Materials and Methods using 0.34 and 1.5 μg of *S. griseus* chitinase protein respectively for the soluble (○) and suspended (●) chitosan reactions. Points in the graph are the mean ± SD ($N = 6$).

RESULTS

Effects of pH on Activity of S. griseus Chitosanase

The pH optimum was determined to be between 5 and 6.5. This was within the range reported for *Streptomyces* sp. 6 (19).

Temporal Studies Using Microbial Chitinase/ Chitosanase Preparations

Typical reaction progress curves that result from the action of *S. griseus* chitinase on chitosan suspensions and soluble chitosan are illustrated in Fig. 1. The reactions proceeded in a linear fashion for at least 35 to 40 min under the conditions specified. The soluble chitosan preparations required less enzyme than the assays using suspended chitosan to yield the same activity.

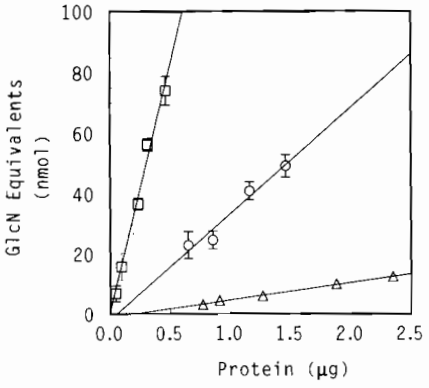

FIG. 2. Dependence of the chitinase and chitosanase reactions on protein concentration. Assays were conducted as described under Materials and Methods. Points depicted in the graph are the mean ± SD ($N = 6$). Protein is shown as μg protein · 300 μl^{-1} reaction mixture. ○, chitosanase reaction utilizing suspended chitosan; △, chitinase reaction; reaction times, 30 min. Chitosanase assay utilizing soluble chitosan, □; reaction time, 20 min.

Protein Dependency of Chitinase and Chitosanase Reactions in S. griseus Preparations

Ohtakara *et al.* (10) have shown that *S. griseus* chitinase acts not only on chitin but also on chitosan; i.e., it possesses both chitinase and chitosanase activities. This presented us with an opportunity to compare the specificities of the enzyme to chitin and chitosan and to compare the relative sensitivities of the chitinase (radioassay) and chitosanase (fluorometric) assays using soluble and suspended shrimp shell chitosan. The protein concentration of the *S. griseus* enzyme was varied in the reactions while the incubation time was held at either 20 or 30 min. The results of these experiments are given in Fig. 2. Under these conditions, the chitinase and chitosanase reactions were linear with regard to enzyme protein concentration between 0.65 and 2.3 μg for 300 μl reaction mixture using suspended chitosan (Fig. 2). When soluble chitosan was used, the linear range for the chitosanase assay was between 0.05 and 0.5 μg enzyme protein per 300 μl reaction mixture. Specific activities at saturating substrate levels were calculated to be 1.24 and 6.4 μmol GlcN · min^{-1} · mg $protein^{-1}$ for the suspended and soluble chitosan preparations, respectively. Chitinase activity was determined to be 0.98 μmol GlcN · min^{-1} · mg $protein^{-1}$. The chitosanase assays are comparable in sensitivity to the radioassay for chitinase.

Detection of Chitinase and Chitosanase Activities in Plant Tissues

Valencia sweet orange callus tissue (nonembryogenic) was used as a source of chitinase and chitosanase to determine if the chitosanase assay was suitable for detecting chitosanase activity in plant tissues (Table 1). Chitinase activity was also determined for comparison purposes. Chitinase activity could be determined in the crude extracts but chitosanase could not because the background fluorescence was too high. Presumably, the high background resulted from low-molecular-weight peptides, amino acids, amino sugars, etc., that did not precipitate in TCA but reacted with fluorescamine. Therefore, the extracts were applied to NAP-G25 desalting columns to eliminate these materials from the assay mixture. The data in Table 1 clearly indicate that chitosanase activity is easily detected after passing callus extracts through small exclusion/desalting columns. This treatment had little effect on total chitinase activity while the specific activity increased about 39%. From the results on desalted extracts (Table 1), total and specific chitosanase activities were about five-fold greater than those observed for chitinase in the Valencia callus tissue.

Effect of Different Sources of Chitosan on S. griseus Chitosanase Activity

Chitosans prepared from crab, krill, shrimp, and "crustacean" shells were tested under *S. griseus* chitosanase reaction conditions to determine if they would affect the chitosanase activity. Chitosanase activities were conducted at two different pH's because of solubility problems with the completely deacetylated preparations of chitosan. The 0% crab chitosan was completely insoluble at pH 6.5 for the soluble assay. The results of these experiments are listed in Table 2. There were dramatic differences in activities depending on the source of chitosan used. There was a greater than 4-fold difference between the shrimp and crustacean chitosan suspension preparations that contained 10–20% acetylation. A 14-fold difference was observed between the 18%

TABLE 1

Chitinase and Chitosanase Activities in Valencia Callus Preparations

Preparation	Total protein (mg)	Chitinase[a] Total activity (U)	Chitinase[a] Specific activity (U/mg protein)	Chitosanase[a] Total activity (U)	Chitosanase[a] Specific activity (U/mg protein)
Crude extract	68.1	1126	16.5	nd	nd
Desalted extract	460	8535	18.6	4113	89.4

[a] Chitinase and chitosanase activities were determined as described under Materials and Methods. Shrimp shell chitosan (soluble; Atomergic Chemetals) was used to determine chitosanase activity.

TABLE 2

Chitosanase Activity of *S. griseus* Chitinase on Different Chitosan Samples

Chitosan source	Chitosanase activity[a]			% Acetylation
	Suspension (pH 6.5)	Solution (pH 6.5)	Solution (pH 5)	
Shrimp	0.56 ± 0.03	7.12 ± 0.74	5.81 ± 0.49	18.0 ± 0.5
Krill	0.35 ± 0.03	5.01 ± 0.75	3.97 ± 0.94	20.1 ± 0.8
Crab	0.14 ± 0.0	4.22 ± 1.24	2.38 ± 0.23	13.8 ± 1.8
Crustaceae	0.12 ± 0.01	2.83 ± 0.18	1.76 ± 0.33	10.5 ± 3.1
Shrimp	0.08 ± 0.01	0.74 ± 0.10	0.43 ± 0.04	0.0 ± 0
Crab	0.04 ± 0.02	nd	0.39 ± 0.07	0.0 ± 0

[a] Reactions were as described under Methods except that 3 μg *S. griseus* chitosanase protein was used and reactions were conducted for either 20 min (solutions) or 1 h (suspensions) at 37°C. The amount of chitosan was 0.4 mg per reaction. Activity is given as the mean (μmol GlcN · min^{-1} · mg $protein^{-1}$) ± SD. The SD is calculated by a mean of four replications. nd, not determined.

acetylated shrimp chitosan and the 0% acetylated crab preparation for the suspension assays. Solubilizing the substrate increased the activity tremendously. Upon solubilization, the activity increased from 12.7-fold for the 18% acetylated shrimp chitosan to 30.1-fold for the 10.5% acetylated crab shell chitosan.

All of the substrates can be compared when they are used either in solution assays at pH 5 or in suspension assays at pH 6.5. On the basis of the activities of the acetylated chitosans with *S. griseus* enzyme preparations, the enzyme substrate preference is shrimp > krill > crab > crustacean. This order roughly parallels the percent acetylation of the chitosan preparations (Table 2). These results suggest that chitosanase activity is related to the degree of acetylation of the chitosan used. Experiments were therefore conducted using completely deacetylated samples of chitosan from shrimp and crab. Very little activity (ca. 0.4 μmol GlcN · min^{-1} · mg $protein^{-1}$) was observed with completely deacetylated shrimp and crab chitosans (Table 2).

Effects of Protein and Proteases

It is possible that proteases and extraneous proteins could create artifacts in the assay by providing substances that can react with fluorescamine. Bovine serum albumin was added (3 to 150 μg protein) to reactions with and without chitosan using both the *S. griseus* and the callus tissue enzyme sources. No differences were observed between the controls (i.e., complete reaction without albumin) and the test reactions containing albumin (data not shown). This indicates that albumin does not affect the chitosanase reactions and that the TCA precipitation efficiently removes contaminating protein. No activity was observed in reaction mixtures without chitosan; this indicates that proteolytic activity was not responsible for the observed activity (data not shown).

DISCUSSION

Our laboratory has had an interest in pathogenesis-related proteins that are resident in *Citrus*. In particular, we have been interested in chitinases and chitosanases because of their potential to reduce losses resulting from fungal invasion by elevating enzyme levels after induction or gene transfers. Although chitosanase appears to be an important member of the pathogenesis-related family of proteins in plants, information on this particular enzyme is sparse in comparison to the literature available on chitinases and β-glucanases. Investigations on chitosanases may have been limited by the lack of facile, sensitive assays.

Given the lack of a suitable chitosanase assay, we set out to develop one. The resulting fluorescence assay utilizes inexpensive and easily available materials and is simple to conduct. Fluorescent derivatives of the chitosanase products are obtained by reaction with the amine reactive substance, fluorescamine. Chitosanase activity is reported in terms of GlcN equivalents using a GlcN standard curve. Fluorescamine reacts with primary and secondary amines, including amino acids, peptides and proteins, and amino sugars (14,20,21). An advantage of this method is that the reagent, fluorescamine, is nonfluorescent and only reaction with primary amines yields a fluorescent product; excess fluorescamine reacts with water to give a nonfluorescent decomposition product (20). Other advantages are that reaction with amines occurs rapidly (in milliseconds) and that small amounts (nanomole to picomole range) of amino sugars (14) and amino acids (20,21) can be detected.

The data presented here indicate the suitability of this assay for measurement of chitosanases from microbial and plant sources. Assay of chitinase and chitosanase in a chitinase preparation from *S. griseus* demonstrates that both enzymes can be detected, supporting the report of Ohtakara *et al.* (10) that *S. griseus* chitinase is active against chitosan.

It is our recommendation that chitosan solutions, rather than suspensions, be used where possible. Chitosan solutions are much easier to manage by pipette and they yield higher activities. Presumably, the higher enzyme activities resulting from the use of chitosan solutions is due to better substrate availability to the enzyme. Results with chitosan suspensions were provided, since assay with insoluble chitosan may sometimes be necessary.

Under our assay conditions, the limits of detection for the chitosanase assay are comparable to those of the chitinase radioassay developed by Molano *et al.* (11). The limits of GlcN detection can be increased substantially by using a larger aliquot of reaction supernatant for reaction with fluorescamine and by increasing the sensitivity of the fluorimeter; a 50-fold increase could be easily obtained. As with most fluorescamine reactions, it is important to maintain the optimum pH for reaction with the primary amine to obtain complete reaction and, hence, maximum sensitivity (22). The optimum pH range for reaction of GlcN with fluorescamine is pH 8–9 (unreported data). Sensitivity may also be increased by increasing the final amount of the fluorescamine carrier solvent in the reaction mixture. Tomkins *et al.* (23) have shown that about a 10-fold increase in sensitivity is obtained in fluorescamine reactions when the amount of fluorescamine carrier solvent (either acetone or acetonitrile) is 50% of the final reaction mixture. This effect is undoubtedly due to solvent interaction with the fluorophore in the excited state (24); solvent interaction with substances during the excited state can dissipate energy and subsequently lower the quantum yields of fluorescent compounds. In addition, reports (23) have indicated that a number of hydroxylic solvents (e.g., alcohols) are unsuitable solvents for fluorescamine because they form additional products with the result that reagent reactivity is lowered.

The chitosan source may also affect the sensitivity of the assay. Our results indicate that large differences in activity can be obtained using different chitosan preparations. Possible explanations for the difference may either be the degree of acetylation remaining in the chitosan preparations or the length of chitosan fibrils as shown by Koga *et al.* (25). Complete deacetylation of chitosans evidently leads to shorter-chain-length molecules (17). The degree of acetylation may vary considerably among different commercial preparation of chitosan (Table 2) (17). We found that the chitosanase activity in *S. griseus* preparations was greatest with commercial chitosan preparations having 18–20% acetylation. Direct comparisons of enzyme activity between different laboratories studying chitosanases will be difficult because of the influence of substrate source. Investigators should include the biological source and the percentage of acetylation of their chitosan preparations so that comparisons might be made. Although we did not compare activities of various chitosanases with those of different chitosan substrates it is likely that large differences in activities would be observed, i.e., that the enzymes from different sources would have different substrate preferences.

Experiments with Valencia sweet orange callus tissue utilizing the fluorescent and radioisotopic assays showed that chitinases and chitosanases were present. This is the first direct evidence for these enzymes in *Citrus*. Gavish *et al.* (26) have reported that antibodies raised against tobacco chitinase and 1,3-β-glucanase cross-reacted with extracellular proteins isolated from sour orange nucellar cell cultures; however, there was no confirmation of activity. On the basis of the activity data, it appears that chitosanase is present in higher concentrations than chitinase. Electrophoretic and isoelectric focusing data indicate that there are several acidic and basic chitinases (unreported). We have not been able to determine whether the chitinases possess chitosanase activity or if there are separate chitosanases; however, incubation of the desalted extracts with completely deacetylated shrimp chitosan resulted in low chitosanase activities (unreported). Recently, Grenier and Asselin (4) reported specific chitosanase isoenzymes in the leaves of barley, tomato, and cucumber.

In summary, a quantitative fluorescence assay for chitosanase has been developed. The suitability of the assay for detection of chitosanase activity has been demonstrated using microbial and plant tissue sources.

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REFERENCES

1. Boller, T. (1985) *in* Cellular and Molecular Biology of Plant Stress (Key, J. L., and Kosuge, T., Eds.), pp. 247–262, Liss, New York.
2. Carr, J. P., and Klessig, F. (1989) *in* Genetic Engineering: Principles and Methods (Setlow, J. K., Ed.), Vol. 11, pp. 65–109, Plenum Press, New York.
3. Monaghan, R. L., Eveleigh, D. E., Tewari, R. P., and Reese, E. T. (1973) *Nature New Biol.* **245**, 78–80.
4. Grenier, J., and Asselein, A. (1990) *Mol. Plant–Microbe Interact.* **3**, 401–407.
5. Legrand, M., Kauffmann, S., Geoffroy, P., and Fritig, B. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6750–6754.
6. Bartnicki-Garcia, S. (1968) *Annu. Rev. Microbiol.* **22**, 87–96.
7. Mayer, R. T., Cunningham, G., and Gupton, J. (1990) *in* Safer Insecticides: Development and Use (Hodgson, E., and Kuhr, R., Eds.), pp. 209–255, Dekker, New York.
8. Chen, A. C., Mayer, R. T., and DeLoach, J. R. (1982) *Arch. Biochem. Biophys.* **216**(1), 314–321.

9. Bedbrook, J. R., Jones, J., Suslow, T., and Dunsmuir, P. (1990) *in* Genetic Improvements of Agriculturally Important Crops: Progress and Issues, (Fraley, R. T., Frey, N. M., and Schell, J., Eds.), pp. 65–68, Cold Springs Harbor Laboratories, Cold Springs Harbor, NY.
10. Ohtakara, A., Matsunaga, H., and Mitsutomi, M. (1990) *Agric. Biol. Chem.* **54,** 3191–3199.
11. Molano, J., Duran, A., and Cabib, E. (1977) *Anal. Biochem.* **83,** 648–656.
12. Nelson, N. (1944) *J. Biol. Chem.* **153,** 375–380.
13. Trudel, J., and Asselin, A. (1989) *Anal. Biochem.* **178,** 362–366.
14. Chen, A. C., and Mayer, R. T. (1981) *J. Chromatogr.* **207,** 445–448.
15. Mayer, R. T., Chen, A. C., and DeLoach, J. R. (1980) *Insect Biochem.* **10,** 549–556.
16. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
17. Domard, A., and Rinaudo, M. (1983) *Int. J. Biol. Macromol.* **5,** 49–52.
18. Murashige, T., and Tucker, D. P. H. (1969) *in* Proceedings, First International Citrus Symposium, Vol. 3, pp. 1155–1161.
19. Price, J. S., and Storck, R. (1975) *J. Bacteriol.* **124,** 1574–1585.
20. Udenfriend, S., Stein, S., Bohlein, P., Dairman, W., Leimgruber, W., and Weigele, M. (1972) *Science* **178,** 871–872.
21. Weigele, M., DeBermader, S. L., Tengi, J. P., and Leimgruber, W. (1972) *J. Am. Chem. Soc.* **94,** 5927–5928.
22. De Barnado, S., Weigele, M., Toome, V., Manhart, K., Leimgruber, W., Bohlen, P., Stein, S., and Udenfriend, S. (1974) *Arch. Biochem. Biophys.* **163,** 390–399.
23. Tomkins, B. A., Ostrum, V. H., and Ho, C.-H. (1980) *Anal. Lett.* **13,** 589–602.
24. Parker, C. A. (1968) Photoluminescence of Solutions, pp. 14–15, Elsevier, New York.
25. Koga, D., Kailka, J., and Kramer, K. J. (1983) *Insect Biochem.* **13,** 295–305.
26. Gavish, H., Vardi, A., and Fluhr, R. (1991) *Physiol. Plant.* **82,** 606–616.